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Activation of Porcine Oocytes Following Intracytoplasmic Injection of Various Sperm Components and foreign species spermatozoa

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여러 가지 정자구성성분 및 이종정자 주입에 의한 돼지난자의 활성

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= 국문초록 =

본 연구에서는 돼지 난자내에 돼지정자, 여러 가지 처리된 정자두부 (1% Triton, 0.1% Trypsin, 100mM NaOH) 및 이종정자 (소, 쥐, 사람)를 미세 주입한 후 난 활성과 웅성 전핵형성, 전핵의 이동 및 배발달을 관찰하였다. 전자현미경으로 관찰한 결과 Triton X-100을 처리하였을 때 첨체가 제거되었으나 핵 주변 물질은 제거되지 않았고 Trypsin 또는 NaOH를 처리 할 경우 핵주변 물질 (perinuclear material)이 제거됨을 볼 수 있었다. 돼지난자는 정자, 정자두부 및 Triton X-100을 처리한 정자두부의 주입을 통해 난 활성이 유도되었으며 쥐, 소, 사람의 정자를 주입하였을 때 난 활성이 유도되고 정상적인 전핵형성이 이루어졌다. 그러나 정자꼬리나 Trypsin 또는 NaOH에 의해 정자 핵주변 물질 (perinuclear material)이 제거된 정자두부를 주입하였을 때는 난 활성은 야기되지 않았다. 유사분열 및 2-세포기까지 정상적인 수정은 동종의 정자 및 정자두부를 주입한 난자에서 관찰할 수 있었으나 이종정자를 주입한 난자에서는 관찰되지 않았다. 또한 상실배 및 배반포까지 정상적인 수정은 동종의 정자 및 정자두부를 주입한 난자에서 관찰할 수 있었다. 이러한 결과는 돼지에서 정자 및 정자두부의 미세주입에 의해 수정이 이루어지는 것을 시사하며 수정시 정자유래의 난활성인자는 정자 핵주변 물질(perinuclear material)에 존재하며 종특이적이지 않다는 것을 보여주는 것이다.

Key Words: Porcine eggs, ICSI, Fertilization, Activation, Perinuclear material

INTRODUCTION

During fertilization the sperm cell activates oocytes by releasing an oocyte activating factor (s) (OAF) into oocytes. Parrington (1996) reported that OAF is a 33 kDa protein residing in the equatorial segment region of the acrosome. Because intracytoplasmic sperm injection of foreign species, such as hamster, rabbit, pig, human or sea urchin into mouse oocytes activates oocytes, OAF is not strict species specific for the mouse oocyte (Wakayama *et al.*, 1997; Kimura *et al.*, 1998). In the mouse OAF appears (or become active) in spermiogenesis and located in the perinuclear material (Kimura *et al.*, 1998). However, information is largely lacking on the nature of activation factors for any species other than the mouse.

To get insight into the nature of sperm born

oocyte factors, we determined the incidence of activation, male pronuclear formation and pronuclear apposition following injection of various porcine sperm components and foreign species spermatozoa into porcine oocytes. In the present study we also examined developmental ability *in vitro* following injection of a spermatozoon and an isolated sperm head.

MATERIALS AND METHODS

1. *In Vitro* Maturation

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25℃ in Dulbecco's phosphate buffered saline supplemented with 5.54 mM D-glucose, 0.33 mM sodium pyruvate, 75 mg/ml potassium penicillin G and 50 mg/ml streptomycin sulphate (mDPBS). Cumulus-Oocyte complexes (COC) were aspirated with an 18-gauge needle into a disposable 10-ml syringe from follicles. Fifty porcine COC were matured in 500 μl of BSA-free NCSU 23 (Petters and Wells, 1993) medium supplemented with 10% porcine follicular fluid, 0.6 mM cysteine, 10 IU/ml PMSG (Sigma, St. Louis, MO, USA) and 10 IU/ml hCG (Sigma) under paraffin oil at 39℃ for 40 to 44 h.

2. Preparation of spermatozoa and isolated sperm heads

Porcine sperm-rich fraction (15 ml) was collected from a boar by gloved hand method, and, after adding antibiotic-antimycotic solution (Sigma), the semen sample was kept at 20℃ for 16 h. Semen was washed 3 times by centrifugation with 0.9% (w/v) NaCl supplemented with 10 mg/ml BSA (fraction V; Sigma). Spermatozoa were suspended in 1.5 ml of TL-HEPES (Prather *et al.*, 1995) for 30 min to induce capacitation. The isolated sperm heads were obtained by the method reported by the Kuretake *et al* (1996). The sperm suspension was sonicated in 9 ml of cold nucleus isolation medium (NIM, Kuretake *et al.*, 1996) in the presence of 0.05% triton X-100. The sonication was conducted in water bath for 30 sec using 100 % output of an ultra sonic sonicator (Model, Branson 8210). The sonicated sperm suspension was diluted and centrifuged for 5 min at 1,000×g to the sperm heads. The sperm heads were once more washed in 10 ml of NIM by centrifugation. For another series of experiments isolated sperm heads were treated with 0.1% trypsin or 100 mM NaOH solution for 3 h in tris buffered saline. Some sperm heads with or without treatments were fixed in 2% glutaraldehyde in Dulbecco's phosphate-buffered saline. The specimens were then post-fixed in 2% OsO_4 for 1 h, dehydrated in a graded ethanol series, and embedded in epoxy resin. Ultrathin sections were cut with a diamond knife and post-stained first with 1% uranyl acetate in 30% ethanol and with Reynolds lead citrate. A transmission electron microscope (Hitachi 600, Hitachi Ltd, Kashiwa, Japan) was used to determine the presence and absence of perinuclear material.

Human ejaculated semen samples were obtained by masturbation. Semen were mixed with an equal volume of HEPES- buffered human tubal fluid medium (HTF-Pepes, Irvine Scientific, Santa Ana, CA) and centrifuge for 5 to 10 min at 1800 g. The pellet was suspended in 1.5 ml of HEPES-buffered HTF-Pepes to induce capacitation. Mouse spermatozoa were collected from a mature male mouse (ICR, 8 to 13 week old) cauda epididymise. Spermatozoa were washed in TL-HEPES and then suspended in 1.5 ml of TL HEPES. Bovine spermatozoa were prepared from frozen-thawed semen. Spermatozoa were washed twice with TL-HEPES and then suspended in 1.5 ml of TL-HEPES. The sperm was resuspended to 1 ml with heparin-containing (10 μg/ml) TL-HEPES in a effendorf tube and kept at 39℃ for 30 to 60 min to induce capacitation.

3. Oocyte Activation

The procedures for electrical stimulation of

porcine oocytes were as described by Kim *et al* (1996a). Electrical stimulation to induce activation was delivered with a BTX Electro Cell Manipulator (Biotechnologies and Experimental Research, Inc., San Diego, CA) to a chamber with two parallel platinum wire electrodes (200 mm o.d.) spaced 1 mm apart overlaid. At 30 to 60 min before sperm cell injection, cumulus cell denuded oocytes were stimulated by a 10 sec pulse at 0.48 KV/cm AC followed by a 30 ms pulse at 1.26 kV/cm DC at room temperature (25℃). The oocytes were then transferred to 500 ml of NCSU23 medium and cultured at 39℃ in an atmosphere of 5% CO_2 in air until sperm cell injection.

4. Spermatozoon injection into Oocytes

Spermatozoa were centrifuged (400g, 5 min) and resuspended in TL HEPES: 10% polyvinylpyrrolidone solution (1:1). A microdrop (5 ml) of this suspension was placed a slide, and the slide was placed in Leitz Differential Interference Contrast inverted microscope equipped with Leitz micromanipulators. The oocytes were denuded of cumulus cells by repeated pipetting. Oocytes with visible polar body and of excellent morphology were used for this experiment. Oocytes were centrifuged for 10 min in an Eppendorf centrifuge at 12,000 g in 50 ml TL-HEPES medium in 1.2 ml Eppendorf centrifuge tube. The injection of spermatozoon or isolated sperm head into the oocyte cytoplasm was performed using the method of Lee *et al* (1998). Briefly, the injection needle used was of 6~7 m inner and 8~9 m outer diameter. The polar body was at 6 or 12 o'clock and the point of injection at 3 o'clock. An oocyte was penetrated by the injecting micropipette, a small amount of cytoplasm was drawn into the micropipette, and then the cytoplasm together with the spermatid and a small amount of medium was expelled into the oocyte. Immediately after ooplasmic injection, the injecting micropipette was withdrawn quickly, and the oocytes were released from the holding pipette to reduce the intracytoplasmic pressure exerted to the oocyte. After injection, all of the oocytes were transferred to NCSU23 medium and cultured for at 39℃ under 5% CO_2 in air. The oocytes were fixed at 10 to 15 h and 20 to 24 h following sperm cell injection. About 10 oocytes were fixed, stained with 1% (w/v) orcein and examined nuclear morphology at 200 X and 320 X magnification with phase contrast microscopy. The others were cultured for NCSU23 medium at 39℃ under 5% CO_2 in air.

5. Chromosomal analysis

At 22 h following injection, the oocytes were cultured in culture medium in the presence of 5 μM colcemid. The oocytes were then centrifuged to remove lipids in the cytoplasm, and placed in a hypotonic solution of sodium citrate (1%) for 5 min and fixed individually on microscope slides by the air-drying method (Tarkowski, 1966). The oocytes were stained with 10% Giemsa at pH 6.8. The chromosome constituent of each spread was determined at 1000 magnification with a Zeiss microscope.

6. Statistical Analyses

The data were pooled from at least four replications. Differences in the percentages of oocytes developing to a particular stage were determined by Chi-square procedures.

RESULT

Fertilization of porcine oocytes following injection of spermatozoa or isolated sperm head

The oocytes with two large pronuclei and two polar bodies (2 PN + 2 Pb) were classified as normal fertilization at 10 to 15 h following ICSI or intracytoplasmic sperm head injection (Table 1). The incidences (52 to 56 %) of normal fertilization were not different between following spermatozoon and sperm head injection. Table 2 summarize chromatin configuration in

Table 1. Chromatin configurations in pig oocytes at 12 to 15 h following intracytoplasmic spermatozoa or isolated sperm head

Parameter assessed	Type of cells injected		
	Sham	Spermatozoon	Sperm Head
No. of oocytes[a]			
Successfully injected (replication)	51 (5)	60 (5)	62 (5)
With two PN and two PB (%)	0 (0)	31 (52)	35 (56)
With one PN, sperm head and two PB (%)	0 (0)	9 (15)	5 (8)
With two PN, sperm head and one PB (%)	0 (0)	2 (3)	4 (6)
With three PN + one PB (%)	2 (4)	5 (8)	6 (10)
With two PN + one PB (%)	9 (18)	1 (2)	0 (0)
With one PN + two PB (%)	36 (71)	0 (0)	0 (0)
With others[b]	4 (8)	12 (20)	12 (19)

[a]Abbreviations: PN, pronucleus; PB; polar body
[b]Others include the oocytes without or unidentified multiple nuclei

Table 2. Chromatin configurations in pig oocytes at 20 to 24 h following intracytoplasmic spermatozoa or isolated sperm head

Parameter assessed	Type of cells injected		
	Sham	Spermatozoon	Sperm Head
No. of oocytes[a]			
Successfully injected (replication)	49 (5)	61 (5)	62 (5)
Closely apposed 2 PN	0 (0)	20 (33)	18 (29)
Mitosis	1 (2)	2 (3)	3 (5)
Two-cell	2 (4)	8 (13)	6 (10)
With two PN and two PB (%)	0 (0)	7 (11)	14 (23)
With one PN, sperm head and two PB (%)	0 (0)	9 (15)	5 (8)
With two PN, sperm head and one PB (%)	0 (0)	2 (3)	4 (6)
With two PN + one PB (%)	10 (20)	0 (0)	0 (0)
With one PN + two PB (%)	27 (55)	0 (0)	0 (0)
With others[b]	9 (18)	13 (21)	12 (19)

[a]Abbreviations: PN, pronucleus; PB; polar body
[b]Others include the oocytes without or unidentified multiple nuclei

porcine oocytes at 20 to 24 h after spermatozoon or isolated sperm head injection. The incidence of pronuclear apposition, mitosis and two cell division was considered as normal fertilized. Following ICSI and head injection, 49% and 44% were normally fertilized, respectively.

Oocyte activation and pronuclear formation following injection of various sperm components

Table 3 summarizes the incidence of activation and male pronuclear formation between 10 to 12 h following injection of various sperm components. When a spermatozoon or Triton X-

Table 3. Activation and male pronuclear formation in porcine oocytes at 10 to 15 h following various sperm components

Sperm heads treated	No. (%) of oocytes		
	Successfully injected(r)	Activated	MPN
None	42 (5)	7 (17)	–
Spermatozoa	25 (4)	19 (76)	15 (60)
Sperm head			
1% triton	46 (5)	41 (89)	29 (63)
0.1% trypsin	44 (5)	10 (23)	6 (14)
100 mM NaOH	35 (4)	3 (9)	0 (0)
Sperm tail	19 (3)	2 (11)	–

MPN: male pronucleus

100 treated porcine sperm heads were injected into porcine oocytes, the oocytes were activated, whereas tail did not induce activation. Injection of either trypsin treated sperm head or NaOH treated spermatozoa failed to induce activation. Male pronucleus was formed in the activated oocytes following injection of Triton X-100 treated sperm head. Neither trypsin nor NaOH treated sperm head was decondensed in porcine oocytes when they were injected. Transmission electron microscopy showed sagittal sections of isolated sperm heads after sonication in the presence of triton X-100 or trypsin (Figure 1). While Triton X 100 treatment left perinuclear material around the nucleus, trypsin or NaOH removed perinuclear material extensively.

Fig 1. Sagittal sections of porcine sperm head following various treatments. **A)** Intact spermatozoa, **B)** Sonicatd sperm head in the presence of triton X, **C)** The sperm head cultured in 100 mM NaOH.

Oocytes activation and male pronuclear formation following injection of foreign species spermatozoa

Most oocytes were activated at 10 to 12 h following injection of sperm cell regardless of electrical stimulation (Table 4). Although some oocytes (23%) were activated in the oocytes following sham injection, the rates is very low. The incidence of activation and pronuclear formation was not different in oocytes following injection of porcine, bovine, mouse or human spermatozoa (Table 4) in the absence of electrical stimulation. Additional electrical stimulation did not increase the incidence of male pronuclear formation ($P>0.1$). At 36 h following injection, majority oocytes, which were injected porcine spermatozoa, were cleaved to the 2-cell stage (Table 5). However, none of oocytes were normally divided to the two cell stage following foreign species sperm injection. Although some oocytes were divided to the two-cell stage following sham, mouse, bovine or human sperm cell injection, it appears to be due to parthenogenetic activation.

In Vitro development of porcine oocytes following injection of a spermatozoon or isolated sperm head

Chromosome analysis revealed that most oocytes among identified them (73% in ICSI; 70% in head injection) were diploid following ICSI and isolated sperm head injection. There is no diploid in oocytes at 22 h after sham injection (Table 6). The most oocytes cleaved at

Table 4. Activation and male pronuclear formation in porcine oocytes at 10 to 15 h following mouse, bovine, human and porcine sperm injection

Source of spermatozoa	Electrical stimulation	No. (%) of oocytes		
		Successfully injected(r)	Activated	MPN
None	+	28 (4)	26 (93)	0 (0)
(sham injection)	−	26 (4)	6 (23)	0 (0)
Pig	+	55 (6)	53 (96)	34 (62)
	−	50 (6)	42 (84)	29 (58)
Cow	+	52 (6)	50 (96)	31 (60)
	−	52 (6)	43 (83)	23 (44)
Human	+	54 (6)	48 (89)	25 (46)
	−	59 (6)	39 (66)	31 (53)
Mouse	+	50 (6)	49 (98)	36 (72)
	−	51 (6)	37 (73)	29 (57)

Table 5. Cleavage and nuclear status of porcine oocytes at 36h following intracytoplasmic injection of porcine, bovine, mouse or human spermatozoa

Source of spermatozoa	No. (%) of oocytes			
	Successfully injected(r)	One cell	Normal two cell[a]	Abnormal two cell[b]
Sham injection	15 (2)	11 (73)	1 (7)	4 (27)
Pig	26 (3)	5 (19)	17 (65)	4 (15)
Cow	27 (3)	17 (63)	0 (0)	10 (37)
Human	25 (3)	16 (64)	0 (0)	9 (36)
Mouse	29 (3)	19 (66)	0 (0)	10 (34)

[a]Embryos with two interphase nuclei
[b]Two to four cell stage eggs with a blastomere which have no or multiple nuclei

Table 6. Chromosomal analysis of porcine oocytes at 22 h following spermatozoon or isolated sperm head injection

Type of cell injected	No. (%) of eggs				
	Tested	Identified	Haploid	Diploid	>Tetraploid
None	22	9	9 (100)	0 (0)	0 (0)
Spermatozoon	26	15	2 (13)*	11 (73)*	2 (13)*
Sperm head	25	10	2 (20)*	7 (70)*	1 (10)*

*$p<0.05$

Table 7. Cleavage of porcine oocytes at 24 and 48 h after spermatozoa or isolated sperm head injection

Times (h) after injection	Type of cell injected	Total no. of oocytes injected	No. (%) of eggs cleaved to			
			1-cell	2-cell	4-cell	Fragmentation
24	none	65	50 (77)	7 (11)	1 (2)	7 (11)
	spermatozoa	65	13 (20)	45 (69)*	6 (9)	1 (2)
	sperm head	65	16 (25)	38 (58)*	8 (12)	5 (8)
48	none	65	15 (23)	32 (49)	6 (18)	12 (9)
	spermatozoa	65	8 (12)	10 (15)	38 (58)*	9 (14)
	sperm head	65	11 (17)	16 (25)	41 (63)*	7 (11)

*$p<0.05$

Table 8. In vitro development of pig zygotes cultured for 7 and 9 days following spermatozoon or sperm head injection

Type of cell injected	No. of embryos examined (r)	No. (%) of oocytes developed to blastocysts	
		at 7 days	9 days of culture
None	65 (5)	2 (3)	8 (12)
Spermatozoon	65 (5)	25 (38)*	29 (45)*
Isolated sperm head	65 (5)	14 (22)*	20 (31)*

*$p<0.05$

24 h and divided to 4- cell at 48 h following ICSI or isolated sperm head injection (Table 7). However, the sham oocytes cleaved to the 2-cell after 24 h. At 7 days the incidences of blastocoel formation following ICSI (38%) and isolated sperm head injection (22%) were higher that following sham injection control (3%, Table 8). Some oocytes following sham injection were developed to blastocysts at 9 days.

DISCUSSION

Intracytoplasmic sperm injection (ICSI) has become a routine clinic procedure for the treatment of male factor infertility. Because injection of a single spermatozoon into an oocyte bypasses contact and fusion between the plasma membrane of both gametes, concerns have been raised how the oocytes can be activated following ICSI. It has been known that in the human direct injection of spermatozoa induce oscillatory pattern of Ca^{2+} rise by introduction of sperm born oocyte activating factors (OAF, probably oscillin, Tesarik *et al.*, 1994). In the mouse, the OAF appeared to be a 33 kDa protein residing in the equatorial segment region of the acrosome (Parrington *et al.*, 1996). It was also known that in the mouse OAF appears (or become active) in spermiogenesis and located in the perinuclear material (Kimura *et al.*, 1998). Our experiment showed that Triton X-100 treated porcine sperm head has ability of activating oocytes. However, neither trypsin treated nor NaOH treated spermaozoa did not activate oocytes. Transmission electron microscopy revealed that perinuclear material is bound tightly to the sperm plasma membrane, and treatment of trypsin or NaOH removed perinuclear material. Therefore, like in the mouse (Kimura *et al.*, 1998), in the pig some substance in perinuclear material, which is firmly attached sperm plasma membrane, may activate porcine oocytes during fertilization or

following ICSI. Our study also showed that intracytoplasmic sperm injection of foreign species such as bovine, mouse or human activated porcine oocytes. Previous results showed that the mouse oocytes are readily activated by injection of hamster, human, rabbit, pig or sea urchin spermatozoa (Rybouchkin *et al.*, 1995; Wakayame *et al.*, 1997, Kimura *et al.*, 1998). Collectively, the substance of oocyte activation seems not to be species specific for the porcine oocyte, like for the mouse oocyte.

During transit of spermatozoa through epididymis, sperm nuclei are very stable by an extensive of cross-linking by disulfide bond of protamines, the sperm specific basic proteins, (Bedford & Calvin, 1974). Following sperm penetration into oocyte cytoplasm, protamins are removed and replaced by histones, and the sperm nucleus was remodeled into pronucleus with assembly of the nuclear envelope. Reduction of the sulfate bond by glutathione (Sutopski *et al.*, 1996) and nucleoplasmin from the germinal vesicle (Philpott *et al.*, 1991; Maeda *et al.*, 1998) seem to play the key role to decondense sperm nucleus and form the male pronucleus. In the present study we demonstrated that the nuclei from various species spermatozoa can decondense and form the male pronucleus in porcine oocytes when they were injected. This result extended in the pig the previous successful pronuclear formation in mice or hamster oocytes following injection of human, pig, sea urchin or fish spermatozoa (Yanagida *et al.*, 1991, Wakayama *et al.*, 1997, Kimura *et al.*, 1998). In the present study the formation of male pronucleus has only been observed in the activated oocytes, suggesting that decondensation and pronuclear formation of sperm nuclei in the oocyte cytoplasm is cell cycle dependant. In hamster and mouse oocytes the activity for the sperm decondensation appears after breakdown of germinal vesicle, and reaches a maximum at metaphase II and disappears following pronuclear formation (Usui and Yanagimachi, 1976; Usui *et al.*, 1997; Maeda *et al.*, 1998). Collectively, mammalian oocytes seems to have non species specific, cell cycle dependent ability to decondense sperm nuclei.

During conventional fertilization, once male and female pronuclei are in close proximity, the nuclear envelops disperse, allowing intermixing of the chromosome. Despite of high percentage of apposition of pronuclei following injection of foreign species spermatozoa, we did not observe formation of syngamy or normal two cell division in porcine oocytes. Relatively little is known yet of the detail of formation in syngamy during fertilization. Apparently, the union of the male and female gametes to form syngamy seems to be controlled by species specific characteristics of the oocyte cytoplasm. Further studies are required to identify factors in oocytes affecting pronuclear union to form syngamy following injection of homogeneous and heterogeneous spermatozoa.

This study demonstrated, for the first time, that porcine oocytes injected either a spermatozoon or an isolated sperm head are capable of developing blastocysts. Although a few sham injected oocytes developed to the blastocysts, the incidence of development is very low. Furthermore the time required to form blastocysts is much longer than that following sperm cell injection. The development to the blastocyst following sham injection is due to parthenogenetic activation. The lower incidence and delayed formation of blastocyst of parthenogetically activated porcine oocytes as compared fertilized one have previously been reported (Cha *et al.*, 1997; Kim *et al.*, 1997b).

SUMMARY

We determined the incidence of activation, male pronuclear formation and apposition of pronuclei in porcine oocytes following intracytoplasmic injection of various porcine sperm components and foreign species spermatozoa,

such as mouse, human or cattle. The porcine oocytes were activated by injection of a spermatozoon or an isolated sperm head. Neither isolated sperm tail nor perinuclear material removed sperm head activated oocytes. Because injection of mouse, bovine or human spermatozoon activated porcine oocytes, the sperm born activation factors is not strict species specific. Male pronuclear formation and pronuclear apposition were observed in the porcine oocytes following injection of porcine, bovine, mouse or human spermatozoa. The electrical stimulation following sperm cell injection did not enhance the incidence of male pronuclear formation nor pronuclear apposition compared with sperm cell injection alone ($p>0.1$). Mitosis and two cell division in some oocytes were observed at 20 to 24 h after injection of porcine spermatozoon. However, none of oocytes following injection of mouse, bovine or human spermatozoa developed to the mitotic metaphase or normally divided to the two cell stage. These results suggested that the oocyte activating factor(s) presented in the perinuclear material and it is not species specific for the porcine oocyte.

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